XXVIII. A QUANTITATIVE STUDY OF SUCCINIC ACID IN MUSCLE.

III. GLUTAMIC AND ASPARTIC ACIDS AS PRECURSORS.

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INTRODUCTION.

THAT formation of succinic, malic and fumaric acids takes place in minced muscle from some unknown precursor has been shown in a previous paper [Needham, 1927]. It was also shown that increased formation of these acids followed under anaerobic conditions upon addition of glutamic and aspartic acids to the mass [Moyle, 1924]. In the present work the action of muscle tissue on these two amino-acids and their possible rôle as precursors of succinic acid in the muscle have been investigated.

The production of one molecule of succinic acid from one molecule of aspartic or glutamic acid must involve the removal of nitrogen in some form. It very soon became clear that this form is not ammonia, and further experiments failed to show any increase in urea, uric acid or amide-nitrogen in the muscle samples during the increased production of succinic acid. Indeed, the amino-nitrogen content of these samples estimated by Van Slyke's method was found to be simply the sum of the amino-nitrogen content of the controls plus the amino-nitrogen of the added glutamic or aspartic acid. If one assumes that the same reactions are taking place in oxygen as under anaerobic conditions, but to a greater extent owing to removal of oxidisable reaction products, it might be expected that in oxygen it would be easier to detect some accumulation of nitrogenous non-oxidisable end-products. Estimations of the oxygen uptake in a Barcroft apparatus showed that, with added glutamic or aspartic acid, the increased oxygen uptake was sufficient to account for complete oxidation to carbon dioxide, water and ammonia of from one-half to the whole of the added amino-acid. Nevertheless, no change in the soluble nitrogen fractions could be found, with the exception of a very slight setting free of ammonia. Two possible explanations of these unexpected results were next considered.

In the first place, the increased oxygen uptake consequent upon adding these two amino-acids occurs (in the case of pigeon muscle) to any extent

only with unwashed muscle, which shows a very high respiration of its own. Washing the muscle with distilled water reduces the oxygen uptake of the control to about one-tenth of the original value, but it reduces still more the excess oxygen uptake in the samples containing amino-acid. The possibility therefore arises that the glutamic and aspartic acids are acting simply as stimulants, bringing about increased oxidation of other substances. These two amino-acids themselves have no specific dynamic action in the body, but it was thought that, if small amounts of glycine or alanine were formed from them, these might stimulate an increase in carbohydrate metabolism in the muscle; or possibly that traces of ammonia split off by increasing cell permeability might bring about an increase in oxidation. Quantitative experiments, however, lent no support to this view, all the other amino-acids tried, and ammonium salts in different concentrations, having no stimulatory, but rather an inhibitory effect on muscle respiration. It is also difficult to suppose that the succinic acid production following addition of these acids is stimulated from some other source. Further, by direct estimation of the free aspartic acid it was possible to show that the amount present had undergone diminution, although the amino-nitrogen of the sample remained constant.

In the second place, it is possible that the amino-group of the glutamic or aspartic acid may combine with some carbon chain in the muscle, succinic acid and a new α -amino-acid thus being formed. This possibility is further considered later.

FORMATION OF SUCCINIC, MALIC AND FUMARIC ACIDS FROM GLUTAMIC AND ASPARTIC ACIDS.

The muscle was always used as rapidly as possible after the death of the animal. In the case of beef, muscle removed from the neck of the bullock immediately after slaughtering was brought at once to the laboratory in a cooled pan; a long cylindrical muscle could then be dissected out with little injury, and this, after cooling to about 5° , was minced. The pigeons were killed by bleeding, and were then placed breast downwards on ice for some minutes before dissecting off the breast muscles. The muscles were placed in beakers surrounded by ice for about 15 minutes and minced when their temperature had fallen to about 5° . The frog muscles were dissected off and cooled before mincing.

The minced muscle was weighed out into equal samples (20–50 g. being used for each), and each sample was suspended in 2 volumes of 1.4 % $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, in a Büchner flask. To certain of the flasks the aspartic or glutamic acid, or a mixture of the two, was then added (the amino-acids having been neutralised with sodium hydroxide); an equal volume of water was added to the controls. The flasks were closed with rubber stoppers, and were all evacuated for several minutes; hydrogen was then introduced. The evacuation and filling with hydrogen was repeated twice more. When beef or

pigeon muscle was used, the flasks were kept about 2–3½ hours at 37°; when frog muscle was used, they were kept at room temperature for about 26 hours. In many of the experiments the vessels, instruments, etc. were all sterilised by autoclaving; it made no difference to the results whether this precaution was taken or not.

The results are set out in Table I.

Table I.

No. of exp.	Material	Acid added g. per 100 g. muscle	Succinic acid mg. per 100 g.	Total acids as mg. succinic acid per 100 g.	Malic acid mg. per 100 g.	Extra acid formed mg. per 100 g.	Averages		
							Acids added	Extra succinic acid	Extra total acids
S1	Beef	0.4 H $\bar{A}$ + 0.4 H $\bar{G}$	83.4	—	—	67.8			
		Control	15.6						
S2	Beef	1.3 H $\bar{G}$	52.8	—	—	28.8			
			45.6	—	—	21.6			
		1.3 H $\bar{A}$	43.0	—	—	19.0			
		Control	24.0						
S3	Beef	0.6 H $\bar{G}$ + 0.6 H $\bar{A}$	146	—	—	121			
			130	—	—	105			
			134	—	—	109			
		Control	26						
			23						
S4	Beef	0.47 H $\bar{G}$ + 0.6 H $\bar{A}$	54.4	—	—	37.0	0.49 H $\bar{G}$ + 0.53 H $\bar{A}$	71.9	
		1.4 H $\bar{G}$	43.2	—	—	25.8	1.35 H $\bar{G}$	25.5	
		Control	17.4				1.3 H $\bar{A}$	21.6	
S5	Beef	0.7 H $\bar{A}$ + 0.7 H $\bar{G}$	—	126	—	110.6			
		Control	—	15.4					
S6	Pigeon	1.0 H $\bar{G}$ + 1.0 H $\bar{A}$	243	—	—	214.8			
		Control	28.2						
S7	Pigeon	2 H $\bar{G}$	—	85.2	—	37.3			
(see N1)		1 H $\bar{G}$ + 0.88 H $\bar{A}$	—	146.0	—	98.1			
		Control	—	(too low)		(too low)			
			47.9						
S8	Pigeon	1.32 H $\bar{A}$	—	136.9	—	89.0			
(see N8)		0.75 H $\bar{G}$ + 0.66 H $\bar{A}$	—	228.2	—	170.3			
S9	Pigeon	0.5 H $\bar{G}$ + 0.5 H $\bar{A}$	—	289.0	46	241.1	0.62 H $\bar{G}$ + 0.58 H $\bar{A}$	—	205.7
		1.0 H $\bar{G}$	—	114.6	0	66.7	1.5 H $\bar{G}$	—	52.0
		1.0 H $\bar{A}$	—	157.0	24	109.1	1.2 H $\bar{A}$	—	99.0
S10	Washed (see N3) pigeon	0.5 H $\bar{G}$ + 0.5 H $\bar{A}$	—	113.2	—	107.2			
		Control	—	6.0					
S11	Washed pigeon	0.5 H $\bar{G}$ + 0.5 H $\bar{A}$	—	188	46.2	182.0	0.5 H $\bar{G}$ + 0.5 H $\bar{A}$	—	144.6
S12	Pigeon + toluene	1.0 H $\bar{A}$	—	256.5	157.0				
		1.0 H $\bar{G}$	—	109.0	0.0				
S13	Frog	0.5 H $\bar{G}$ + 0.5 H $\bar{A}$	—	181	45	152.5			
		1.0 H $\bar{G}$	—	95.5	0	67.0			
		1.0 H $\bar{A}$	—	151.0	50	122.5			
		Control	—	28.5					
S14	Washed frog	0.5 H $\bar{G}$ + 0.5 H $\bar{A}$	—	75.5	17.5	37.0			
		1.0 H $\bar{G}$	—	47.0	0	8.5			
		1.0 H $\bar{A}$	—	35.4	0	0			
		Control	—	38.5					

Exps. S1, S2, S3, S4 and S6 were carried out by the earlier method for succinic acid only. In the other experiments the three acids together (succinic, malic and fumaric) were obtained as silver salts; the silver was estimated,

and the result is expressed in the fifth column as succinic acid. The malic acid was also estimated separately in some cases. It is to be supposed that this malic acid has been formed, under the influence of fumarase, by hydration of fumaric acid, and that it is in equilibrium with the latter; as the equilibrium mixture consists of about 70 % malic and 30 % fumaric acid, about one and a half times the succinic acid equivalent of the malic acid figures must be subtracted from the total acid figures to obtain the true succinic acid values. The control value of Exp. S7 is used also for S8 and S9 in calculating the extra acid formed; the control value of S10 is used also for S11.

In all the experiments it appears very definitely that the addition of glutamic and aspartic acids together to the same muscle sample has a greater effect in increasing the succinic acid production than addition of an equivalent amount of either acid alone. The reason for this is not clear. Possibly two enzyme systems are concerned, one with glutamic acid and the other with aspartic acid breakdown; the fact that with washed frog's muscle aspartic acid is quite inactive, while glutamic acid gives rise to a marked succinic acid production and oxygen uptake, also suggests two separate systems. If each system is saturated when only 0.5 g. of the amino-acid is added to 100 g. muscle, then adding 1 g. would have no greater effect, and one would expect to obtain, by adding 0.5 g. of each to the same sample, the sum of the results obtained by adding 0.5 g. of each to two different samples. Actually, rather more than the sum is obtained; the simple hypothesis breaks down particularly, however, in the case of the washed frog's muscle, where, while aspartic acid alone is quite untouched, aspartic and glutamic acids together not only give a greater yield of total acids than does glutamic alone, but give a high percentage of malic acid. The figures obtained are not numerous enough for a detailed analysis of the results of adding the acids separately and together, or for the explicit formulation of any theory of multiple enzyme systems.

The highest yield of succinic acid obtained from the mixed amino-acids corresponds to about 37 % of the added amino-acids; with glutamic acid alone it corresponds to about 11 %, and with aspartic acid alone to about 15 %. When the malic acid is estimated, it is found to appear only after addition of aspartic acid or of aspartic and glutamic acids together; with glutamic acid alone no malic acid production could be found. Quastel and Woolf [1926], working with *B. coli*, showed that aspartic acid gives rise to ammonia and fumaric acid. In absence of growth inhibitors such as toluene, the fumaric acid is reduced to succinic; but the presence of toluene inhibits this reduction. It was now similarly found with muscle (see Exp. S12) that in presence of toluene, aspartic acid gave rise to a much higher percentage of malic acid—about 54 % instead of 20 %. The experiment was performed exactly as usual, except that to the buffer solutions 10 % by volume of toluene was added. Succinic acid only was obtained by adding glutamic acid in this experiment as in the others. It seems, then, that in the muscle succinic

acid is formed directly from glutamic acid, while in the case of aspartic acid, fumaric and malic acids first appear, and are then reduced by reducing substances in the muscle to succinic acid.

In Exps. S10, S11 and S14, the muscle was washed (by shaking gently for 20 minutes in 10 volumes of ice-cold distilled water), and then strained through muslin. (The results given are per 100 g. of muscle before washing; the weight sometimes differed slightly after the process.) In the case of the pigeon, washing did not very greatly inhibit the production of succinic, malic and fumaric acids from glutamic and aspartic, although the muscle alone showed practically no production; with the frog, production from the muscle alone seemed to be unaffected, and some increased production took place from added glutamic, and from the two amino-acids together, but not from aspartic acid.

Table II.

No. of exp.	Material	Acid added g. per 100 g. muscle	Total acids as mg. succinic acid per 100 g.	Malic acid mg. per 100 g.
O1	Pigeon	2 H $\bar{A}$ + 2 H $\bar{G}$	49.6	36
		Control	10.8	2
O2	Pigeon	2 H $\bar{A}$ + 2 H $\bar{G}$	27.2	12
		Control	9.28	—
O3	Pigeon	5 H $\bar{S}$	3.070	2.700
		5 H $\bar{F}$	3.070	2.800
		3 H $\bar{G}$ + 3 H $\bar{A}$	350	370
O4	Pigeon (washed)	5 H $\bar{S}$	3840	3400
		5 H $\bar{F}$	3840	3600
		5 acetic acid	30	

A few experiments were also carried out on the progress of events in oxygen (Table II). Here small but definite amounts of the total acids were obtained; in the case of the controls these seemed to consist almost entirely of succinic, but in the samples to which glutamic and aspartic acids had been added, they consisted partly or wholly of malic and fumaric acids. In Exp. O1 the muscle was suspended in 4 volumes of 1 % NaCl at 35° for 6 hours; oxygen was bubbled through the bottles, which were shaken at intervals. In Exp. O2 the conditions were the same except that the bottles were filled with oxygen and shaken on a shaking machine; at the end of the experiment the muscle appeared brown and coagulated, perhaps owing to too violent shaking. In Exp. O3 the muscle was suspended in 5 volumes of 1.4 % phosphate, the bottles were filled with oxygen and shaken very gently in a Barcroft-bath. In order to compare what happened in similar circumstances with succinic and fumaric acids, these acids also were added to certain samples. With the unwashed muscle it is seen that, in both cases, about 60 % of the original amount added is recovered. As the method gives results 85 % of the theoretical, this means that 70 % remains after the oxidation time and about 30 % has been oxidised away. This 70 % consists entirely of

malic and fumaric acids. With the washed muscle, 77 % (corrected, 90 %) is recovered, and this also consists entirely of malic and fumaric acids.

The free acid was in several cases prepared from the silver salts, as described in an earlier paper [Moyle, 1924]. The acid obtained, after addition of glutamic and aspartic acids, gave a sharp melting-point at 180–181°, when the method including permanganate oxidation was used. When the method giving the total silver salts was used, the acid prepared from these showed partial melting at 180°, which was not complete until 200°. Its behaviour resembled that of a mixture of succinic and fumaric acids. A sample of these "total acids" was tested for succinic acid in the following way. It was treated with five times its weight of *p*-toluidine, and heated for half-an-hour at 220°. The resulting mixture was treated with 50 % alcohol, and the crystalline precipitate of succinotoluidide was filtered off and washed. It was recrystallised from boiling alcohol, washed and dried at 100°; M.P. 258°.

THE FATE OF THE AMINO-GROUP.

The attempts to find changes in the soluble nitrogen fractions during the production of succinic, malic and fumaric acids from glutamic and aspartic acids are summarised in Table III.

Table III.

No. of exp.	Material	Acid added g. per 100 g.	Ammonia-N mg. per 100 g.	Urea-N mg. per 100 g.	Uric acid mg. per 100 g.	Amide-N mg. per 100 g.	
N1	Pigeon	1.0 HG + 0.88 HA	14.5	0			
(see S7)	(anaerobic)	Control	13.0	0			
N2	Pigeon	1.0 HG + 1.0 HA	16.5	0	2.75		
	(anaerobic)	Control	16.5	0	2.12		
N3	Washed pigeon	1.0 HG + 1.0 HA	0.2	0	—	12	
(see S10)	(anaerobic)	Control	0.2	0.2	—	11	
N4	Pigeon	1.2 HG + 15.2 mg. ammonia-N	26.1	—	—	—	85 % added NH ₃ recovered; none formed from HG
	(anaerobic)	Control	13.2				
N5	Pigeon	0.6 HG + 0.5 HA + 13.7 mg. ammonia-N	21.8	—	—	—	75 % added NH ₃ recovered; none formed from HG and HA
	(anaerobic)	Control	11.5				
N6	Pigeon	0.66 HG + 0.66 HA	20.6	—	—	—	6 mg. per 100 g. NH ₃ -N formed
	(oxygenated)	Control	14.7				

The ammonia and urea were estimated in the first experiments (N1, N2 and N3) by Folin and Wu's [1919] method; in the later experiments (N4, N5 and N6) the ammonia was estimated by a modification of Stanford's method [1923]. The entire sample (about 2.5 g.) in 2 vols. of phosphate solution was washed into the distilling flask; saturated borate solution was used instead of soda. The vacuum distillation was carried out at 45° and 15 mm. pressure; for 5 minutes the apparatus was shut off from the pump, then for 5 minutes connected with it. For 10 minutes a very slow stream of

ammonia-free air was drawn through. Under these conditions, with the original volume of liquid in the flask about 30 cc., 80 % of the ammonia present could be estimated. This yield is low but quite constant, and the method has the advantage of working at a comparatively low temperature.

When an extract of the muscle was used, in order to make sure of extracting the ammonium salts without driving off any ammonia, the samples were either (1) treated with alcohol containing enough HCl to bring the final p_H to about 6.0, the alcohol being afterwards removed; or (2) treated with dilute HCl to bring to p_H 6.0–6.5, and coagulated by plunging the vessels into a boiling water-bath. This technique was only used if the samples were small, and therefore could be almost instantaneously coagulated by heat.

Uric acid was estimated by Benedict and Franke's method [1922], using the slightly acid (p_H 6.0) aqueous extract. This was neutralised and evaporated and traces of protein were filtered off.

For the amide estimations the samples after incubation were washed into round-bottomed flasks and rapidly coagulated in a boiling water-bath. HCl was added to make the concentration 5 % by volume, and the contents of the flasks were hydrolysed for 2 hours, under reflux condensers. At the end of this time they were partly neutralised with Na_2CO_3 . The ammonia present was estimated by Folin and Wu's method, and the amide-nitrogen found by subtracting the ammonia present before hydrolysis.

Table IV.

No. of exp.	Material	Acids added g. per 100 g.	Van Slyke-N mg. per 100 g.	Average values mg. per 100 g. for amino-N of muscle alone
N 7	Pigeon (anaerobic) (oxygenated)	0.44 $H\bar{G}$ + 0.44 $H\bar{A}$ (amino-N 88.8 mg.)	143	52.7
		Control	145	
		0.44 $H\bar{G}$ + 0.44 $H\bar{A}$ (amino-N 88.8 mg.)	139	
		Control	138	
N 8 (see S 11)	Washed pigeon (anaerobic) (aerobic)	0.54 $H\bar{G}$ + 0.54 $H\bar{A}$ (amino-N 108.5 mg.)	133.6	15
		Control	116.4	
		0.54 $H\bar{G}$ + 0.54 $H\bar{A}$ (amino-N 108.5 mg.)	117	
		Control	128	
N 9	Washed pigeon (oxygenated)	0.48 $H\bar{G}$ + 0.48 $H\bar{A}$ (amino-N 96 mg.)	114.4	18
		Control	113.0	
N 10 (see B 2)	Washed pigeon (aerobic)	0.5 $H\bar{G}$ + 0.5 $H\bar{A}$ (amino-N 100.5 mg.)	94	13.5
		Control	122	
		$H\bar{A}$ 1 g. (amino-N 105 mg.)	129.8	
		Control	118.0	
N 11 (see A 3 and B 19)	Pigeon (aerobic)	1 g. $H\bar{A}$ (amino-N 105 mg.)	175.8	69.3
		Control	172.8	
N 12	Pigeon (oxygenated)	1 g. $H\bar{A}$ (amino-N 105 mg.)	179.8	67.9
		Control	166.0	

For the estimations of free amino-nitrogen a modification of Parnas and Wagner's procedure [1914] was used. The samples were first coagulated in a boiling water-bath; they were cooled in ice, and HCl was added to make the concentration 0.25 % (by volume). The flasks stood at 0° overnight; next

day the muscle was strained off, ground with more 0.25 % acid, and strained again. The grinding and straining was repeated once more; the filtrate was filtered through paper, neutralised and evaporated. It was centrifuged, and the clear centrifugate was made up to a known volume, of which parts were used for estimations in the Van Slyke apparatus. In some cases, any soluble protein was removed by adding trichloroacetic acid to a concentration of 4 % before centrifuging. This treatment did not seem to affect the results.

In all the experiments in which nitrogenous fractions were to be estimated, the p_H of the buffer solution in which the muscle samples were suspended was 7.0–7.4; 1.4 % $Na_2HPO_4 \cdot 2H_2O$ was taken and brought to the required p_H by adding phosphoric acid. For the anaerobic and some of the aerobic experiments, 2 volumes of buffer solution were used; for the later aerobic experiments (N9, N12), 5 volumes were used. In all the aerobic experiments the vessels used contained a large volume of air or oxygen, which was renewed at intervals, and the vessels were shaken gently. The experiments lasted from $3\frac{1}{2}$ to 5 hours at 37° .

Exp. N1 was performed on the same material as S7; N3 on the same material as S10, and N8 on the same material as S11. For Exp. N10 the material from the Barcroft cups of B2 was used, and for Exp. N11 the material from B19.

No increase in ammonia could be shown under anaerobic conditions, in washed or unwashed muscle. The value obtained, about 14 mg. per 100 g., agrees satisfactorily with the maximum found for pigeon muscle by Parnas and Mozolowski [1927]. Control experiments showed that small amounts of ammonia, such as those equivalent to the amounts of extra succinic acid formed in the samples, added (as $(NH_4)_2SO_4$) to muscle could be quantitatively recovered. It was thought possible that, in presence of excess of glutamic and aspartic acids, there might be combination between ammonia and these acids, whether as amide or in some other form. The ammonium salt was therefore added in presence of the glutamic and aspartic acid, but in these circumstances also could be quantitatively recovered.

In oxygen a small liberation of ammonia took place; it amounted only to about 5 % of that contained in the added amino-acids.

No increase in urea, uric acid or amide-nitrogen was found.

In the Van Slyke experiments, the same amounts of glutamic and aspartic acids were added to the control samples immediately before the flasks were washed out into the coagulation vessel. In some cases, the amino-nitrogen was estimated on the muscle which had been in the Barcroft apparatus, and of which the actual oxygen uptake was known. No significant difference could be found between the results from the samples incubated with the amino-acids (either with or without oxygen for several hours), and those to which the amino-acids were added at the last moment. Control experiments showed that these two amino-acids are quantitatively extracted and estimated by this method, and the values obtained (by subtraction) for the

amino-nitrogen of the muscle alone closely resemble those found by Van Slyke [1913-14] for dog muscle and by Parnas and Wagner [1914] and Meyerhof [1924] for frog muscle.

OXYGEN UPTAKE ON ADDING GLUTAMIC AND ASPARTIC ACIDS.

The measurements of oxygen uptake were made with Barcroft differential manometers and the results are given in Table V. The oxygen uptake of the muscle alone was measured by placing in one cup 1.5 cc. buffer solution and 1.5 cc. water; in the other, 1.5 cc. buffer solution, 0.5 g. of muscle and 1 cc. of water. To find the increased uptake due to addition of the amino-acids, 1.5 cc. of buffer solution and 0.5 g. of muscle were placed on each side, with 1 cc. of water on one side and 1 cc. of the amino-acid solution (neutralised with NaOH) on the other. The muscle was added to the solutions at the last moment before the cups were attached; the apparatus was shaken in the bath for 15 minutes with the taps open. The taps were then closed, and readings were made at intervals. In the earlier experiments, 10 drops of 40 % potassium hydroxide were placed in the central cup for absorbing carbon dioxide; in later experiments, on the advice of Dr M. Dixon, a roll of filter paper soaked in 5 % KOH was placed instead in the cup, and projected from it. The bath was kept at 37° for the pigeon muscle, and at 20° for frog muscle. The oxygen uptake of the pigeon muscle alone fell off rapidly after about 3½ hours; by the time an almost steady state was reached the oxygen taken up usually amounted to about 1800 mm.³ for 0.5 g. muscle, although with some samples it was as high as 2400, and with others as low as 500, for no obvious reason. The increased uptake found in the presence of glutamic and aspartic acids usually varied with the respiratory activity of the muscle alone, being greater if the control respiration was high and lower if the control respiration was feeble. The calculated oxygen required for oxidation of 5 mg. glutamic acid to ammonia, carbon dioxide and water is 3330 mm.³; for 5 mg. of aspartic acid it is 2220 mm.³; for half quantities of each, 2775 mm.³ When 5 mg. of the amino-acid was added, the increased oxygen uptake during the time of the experiment usually amounted to about 1500 mm.³, two-thirds to one-half of the calculated maximum. The extra uptake usually began very slowly, and after about 1 hour increased rapidly, to fall off again in another 2 hours. When the experiment was stopped, the uptake usually continued, but at a very slow rate. When 10 mg. of acid was added, about the same oxygen uptake occurred in the time, but from the shape of the curve was obviously quite incomplete. When 1 mg. was added, oxygen uptake practically came to a standstill by the end of the experiment. Here there was usually enough oxygen absorbed to account for complete oxidation, and sometimes even larger quantities were absorbed (see Figs. 1 and 2). These last experiments may not be very reliable; the oxygen uptake of the muscle alone is so much greater than the increase caused by addition, that small

Table V.

No. of exp.	Tissue	Acids added mg.	Total amount of extra O ₂ uptake mm. ³	Control O ₂ uptake mm. ³	Duration hrs.
B1	Washed pigeon (10 vols. 20 min.)	2.5 H $\overline{G}$; 2.5 H $\overline{A}$ 5 H $\overline{G}$ 5 H $\overline{A}$ Control	68.2 13.1 9.5 —	104.2	4½
B2 (see N10)	Washed pigeon (10 vols. 1½ min.)	2.5 H $\overline{G}$; 2.5 H $\overline{A}$ 5 H $\overline{G}$ 5 H $\overline{A}$ Control	272.5 114.4 286.0 —	284.4	4
B3	Pigeon	2.5 H $\overline{G}$; 2.5 H $\overline{A}$ 5 H $\overline{G}$ Control	1478 1418 —	1905	3½
B4	Pigeon	5 H $\overline{A}$ 1 H $\overline{A}$ Control	1257 637 —	861	4½
B5	Pigeon	5 H $\overline{G}$ Control	2072 —	2376	4½
B6	Pigeon	5 H $\overline{A}$ 1 H $\overline{A}$ Control	518 336 —	561	
B7	Pigeon	10 H $\overline{G}$ 5 H $\overline{G}$ 1 H $\overline{G}$ Control	1743 1673 709 —	2013	5½
B8	Pigeon	1 H $\overline{A}$ 1 H $\overline{G}$ Control	747 452 —	1570	3½
B9	Washed pigeon (10 vols. 20 min.)	5 H $\overline{A}$ 5 H $\overline{G}$ Control	49.1 35.2 —	194	
B10	Pigeon	0.5 H $\overline{G}$ + 0.5 H $\overline{A}$ Control	636.0 —	1848	5½
B11	Frog washed (10 vols. 20 min.)	5 H $\overline{A}$ 5 H $\overline{G}$ 2.5 H $\overline{G}$ + 2.5 H $\overline{A}$ Control	0 194 127.5 —	30.6	5
B12	Frog washed (10 vols. 20 min.)	3 H $\overline{G}$ 2 H $\overline{G}$ 1 H $\overline{G}$ Control	246 182.8 147.5 —	57	7
B13	Pigeon washed (10 vols. 20 min.)	5 H $\overline{G}$ 5 H $\overline{A}$ 2.5 H $\overline{G}$ + 2.5 H $\overline{A}$ Control	285.5 87.0 123.5 —	414	5½
B14	Pigeon washed (10 vols. 20 min.)	5 H $\overline{G}$ 5 H $\overline{A}$ 2.5 H $\overline{G}$ + 2.5 H $\overline{A}$ Control	31.7 0 0 —	146.0	
B15	Frog washed*	5 H $\overline{G}$ 5 H $\overline{A}$ Control	32.9 0 —	0	6
B16	Pigeon washed*	+ 5 H $\overline{G}$ + 5 H $\overline{A}$ Control	24.7 0 —	59.2	
B17	Pigeon	5 H $\overline{G}$ 5 H $\overline{A}$ Control	1917 1626 —	2495	
B18	Frog	1 H $\overline{G}$ 0.5 H $\overline{G}$ Control	245.2 110.1 —	180.5	19½
B19	Pigeon	5 H $\overline{A}$	1399	—	4½

* Six times with 10 volumes of phosphate solution (48 g. Na₂HPO₄, 2H₂O + 54 g. KH₂PO₄ per litre) [see Hahn and Haarmann, 1928].

Fig. 1.

- Uptake of control.
- Extra uptake with 5 mg. glutamic acid.
- ×—×— Extra uptake with 10 mg. glutamic acid.
- ⊙—⊙— Extra uptake with 1 mg. glutamic acid.

Fig. 2.

- Uptake of control.
- Extra uptake with 5 mg. succinic acid; 5 mg. more added at ↓.
- ×—×— Extra uptake with 5 mg. glutamic acid; 5 mg. more added at ↓.
- ⊙—⊙— Extra uptake with 5 mg. aspartic acid. Exp. B 10.
- Uptake of control.
- ⊙--- Extra uptake with 0.5 mg. aspartic acid + 0.5 mg. glutamic acid.

variations in the amount of muscle tissue in the different cups, or small variations in the degree of chopping, might have large effects.

Attempts to reduce the control respiration without too much diminishing the effect of adding the amino-acids were unsuccessful in the case of the pigeon; the oxidation of the added amino-acid was usually reduced to a greater extent than the control respiration, and in some experiments was almost absent. Addition of the two amino-acids together seemed to have no greater effect than addition of either alone with the washed or unwashed muscle.

Fig. 3.

- I. Uptake of control.
- II. Extra uptake with 1 mg. glutamic acid added.
- III. Extra uptake with 2 mg. glutamic acid added.
- IV. Extra uptake with 3 mg. glutamic acid added.

Frog muscle (as was suggested to me by Dr D. C. Harrison) proved much more satisfactory material for a study of the oxygen uptake. By washing once with 10 volumes of distilled water the control respiration falls to about 40 mm.³ for 0.5 g. at 20° for 7 hours, while the increased uptake due to the added amino-acid is five or six times this amount. With washed frog muscle, aspartic acid had no effect. The rate of oxygen uptake varied according to the concentration of added glutamic acid, but the relative absorption was greatest with the smallest amounts (see Fig. 3). The highest was 3 atoms of oxygen per molecule when 1 mg. of glutamic acid was added.

OXYGEN UPTAKE WITH OTHER AMINO-ACIDS AND AMMONIUM SALTS.

In Table VI are given the results of adding some other amino-acids and also ammonium chloride to unwashed pigeon muscle. With 1.75 mg. ammonium chloride, nearly 30 % inhibition of the muscle's own respiration was found; with a tenth of this amount only slight inhibition took place. In these two experiments an equivalent amount of sodium chloride was placed in the opposite Barcroft cup. Glycine, alanine, valine, leucine and tyrosine all showed inhibitory action, sometimes to a very marked degree (see Fig. 4). Glutamic and aspartic acids thus seem to occupy a very special position with regard to muscle.

Table VI.

No.	Material	Substances added	Oxygen uptake	Inhibition %	Duration hrs.
B20	Pigeon	NH ₄ Cl (1.75 mg.)	1760	27	5
		Control	2274		
B21	Pigeon	NH ₄ Cl (0.18 mg.)	817	5	4
		Control	861		
B22 (see B8)	Pigeon	1 mg. glycine	1381	12	4
		Control	1570		
B23	Pigeon	5 mg. alanine	1433	33	3½
		1 mg. alanine	1743	18	
		Control	2125		
B24	Pigeon	5 mg. leucine	890	35	5½
		5 mg. valine	448	67	
		Control	1378		
B25	Pigeon	5 mg. leucine	983	9	4½
		5 mg. valine	886	18	
		Sat. sol. tyrosine	799	26	
		Control	1080		

Fig. 4.

- I. Uptake of control.
- II. Uptake after addition of 1 mg. alanine.
- III. Uptake after addition of 5 mg. alanine.

DISAPPEARANCE OF ADDED ASPARTIC ACID.

These experiments were carried out in order to ascertain whether, under conditions such that a marked increase in oxygen uptake would occur, the added aspartic acid showed any corresponding decrease in amount.

I am indebted to Mr B. Woolf for the suggestion that the aspartic acid might be estimated by conversion into malic acid, as a method was available

for estimation of the latter in muscle. A large number of preliminary experiments showed that with NaNO_2 and acetic acid at room temperature or higher deamination was complete, but a large part of the equivalent malic acid had disappeared, presumably by oxidation. The best results were finally obtained in the following way. 10 cc. of the neutral solution containing aspartic acid cooled to 0° were treated with 1 cc. 30 % NaNO_2 and 0.25 cc. 50 % H_2SO_4 . The mixture was kept at 0° over night, being shaken well at frequent intervals during the first hour. Deamination was then found to be practically complete, and 80 % of the calculated malic acid was found.

The experiments were carried out in the following way. Five g. samples of minced muscle were suspended in 25 cc. of 1.4 % phosphate in bottles of 250 cc. capacity. One bottle (A) contained 50 mg. of aspartic acid. All were shaken very gently, in a horizontal position, at 37° for $4\frac{1}{2}$ hours. The bottles were opened for re-aeration every half hour. At the end of the time one bottle (C +) received 10 cc. solution containing 50 mg. aspartic acid, and 10 cc. water was added to the third (C). Each sample was immediately washed into a boiling-tube, and coagulated in a boiling water-bath. The slightly acid extract was prepared, neutralised and concentrated as in the Van Slyke experiments. To the clear solution, measuring 10 cc., 1 cc. NaNO_2 and 0.25 cc. 50 % H_2SO_4 were added at 0° , as described. The next day the excess of nitrous acid was removed by adding 0.3 g. of urea, and shaking till effervescence had subsided. The solutions were neutralised and made up to 25 cc. They were placed in covered flasks on a boiling water-bath; 5 g. uranium acetate was added, and the flasks were heated for 20 minutes. The contents were cooled in the dark and filtered. The rotation was taken, using the green line of the mercury lamp. Control experiments with malic acid solutions containing phosphate and treated with uranium acetate in this way gave $[\alpha]_{5461}^{20} = -430^\circ$.

Experiment A1.

	Readings	Change in rotation	% recovered
Zero	1.4°		
C	1.5		
C +	0.37	1.13	64
A	0.95	0.55	32

These figures show that only about half the aspartic acid is present after the incubation.

An attempt was made to get conditions on the large scale more resembling those in the Barcroft cups. Five g. samples of muscle in 25 cc. of solution were placed in litre Roux bottles, the flat surface of which was about 220 sq. cm., or ten times the area of the bottom of a Barcroft cup. A stout wire passed through the rubber stopper of each Roux bottle, holding in the centre of the bottle a porcelain boat. The boat contained filter paper soaked with 5 % KOH . In order to promote oxidation, the bottles were filled with oxygen before the experiment began, then they were all placed horizontally

in a bath at 37°, attached to an overhead bar, which moved gently backwards and forwards. The final solutions were made up to 15 cc.

Experiment A2.

	Reading	Change in rotation	% recovered
Zero	3.24°		
C	3.30		
C1 +	1.48	1.82°	63
C2 +	1.60	1.7	61
A	2.10	1.2	42

Here about two-thirds of the aspartic acid remain.

In a third experiment the procedure was exactly as just described, but the muscle was chopped with fine scissors as for the Barcroft experiments, instead of being minced. A sample was used for oxygen uptake estimation (see B19), and the contents of the Barcroft cups were used afterwards for Van Slyke estimations (see N11). The solutions were made up to 15.5 cc., and sodium light was used. $[\alpha]_D^{20}$ was found under these conditions to be -350° . The solution is acid, and the value agrees with that (-345°) found by Itzig [1901] for the compound $\text{UrO}_3\text{C}_4\text{H}_6\text{O}_5$. Addition of alkali, as observed by Walden [1897], increases the rotation to a maximum; almost the same maximum (-512°) was found by Walden for the compound $\text{UrO}_3\text{C}_4\text{H}_5\text{O}_5\text{Na}$.

Experiment A3.

	Reading	Change in rotation	% recovered
Zero	3.4°		
C1 +	5.0	1.6°	71
C2 +	4.9	1.5	66
A1	4.4	1.0	44
A2	4.3	0.9	40

Here about three-sevenths of the aspartic acid has disappeared; the oxygen uptake observed in the time was 1399 mm.³, or almost two-thirds of the amount needed for complete oxidation; the amino-nitrogen was unchanged.

DISCUSSION.

The results described in this paper make it seem very probable that, if free glutamic and aspartic acids are present in the muscle or are brought to it in the blood stream, then production of succinic, fumaric and malic acids must follow, and also an increase in oxygen uptake, probably due to oxidation of these three acids (although there is no evidence that all the glutamic or aspartic acid oxidised passes through the stage of a 4-carbon non-amino-acid).

A comparison of the oxygen uptake curve obtained when glutamic or aspartic acid is added to unwashed pigeon muscle with that obtained when succinic acid is added, shows that the two curves are very similar (see Fig. 2). The chief differences are, firstly, that in the latter case the uptake persists rather longer, and practically the calculated total is absorbed; and secondly that, when the absorption has come to a standstill, it is possible, by adding

more succinic acid, to call forth another marked oxygen absorption, while addition of glutamic acid has no effect. This suggests that the mechanism responsible for the conversion of the amino-acid into succinic or fumaric acid gradually fails, and that under the conditions of the experiment it has failed before complete conversion has had time to take place. If we now consider the aerobic experiments on the large scale, we find, in the first place, that less aspartic acid disappeared (only one-half to one-third) than one would expect from the Barcroft experiments (one-half to two-thirds). Secondly, in Exp. O3, with added succinic acid, a large quantity of fumaric and malic acids appeared at the end of the experiment, while, from the same amount of glutamic and aspartic acids, only about one-ninth of the amount of fumaric and malic acids appeared. These facts might again be explained by the comparatively early failure of the enzyme system producing succinic and fumaric from glutamic and aspartic acids; conditions on the large scale are probably less favourable than in the Barcroft cups. The succinic and fumaric acids formed during the early period of the experiment would gradually be oxidised further. One must not, however, lose sight of the possibility that, even under aerobic conditions, only about 25 % of the amino-acids passes through succinic acid, the rest following some other path of breakdown.

As regards the possible occurrence of the amino-acids in question in the free state in muscle, Osborne and Heyl [1908] for chicken muscle and Osborne and Jones [1909] for ox muscle have shown that glutamic acid constitutes about 16 % of the protein and aspartic acid about 4 %. Micko [1908] found free glutamic acid, alanine and taurine in meat extract, but figures for the free amino-acids of fresh muscle do not seem to be available. There is 0.07 % of amino-nitrogen present in the muscle, and this is probably largely contained in free amino-acids; for the nitrogen of creatine and methylguanidine is not estimated by Van Slyke's method [Van Slyke, 1911], and there are only traces of carnosine present in pigeon muscle [Clifford, 1921]. The fact that washed frog muscle gives the same sort of yield of succinic acid as unwashed muscle, while formation of succinic acid does not take place in washed pigeon muscle (although it retains the power to act on added glutamic and aspartic acids), may be due to the greater acidity developed in a short time by the pigeon muscle. This might enable the content of amino-acids to be washed out of the pigeon muscle, while in the frog muscle they might not be extracted. The fact that, in the silver salts obtained from the muscle alone, malic acid was either absent or present only in traces, may point to the origin of the succinic acid from glutamic acid alone, unmixed with aspartic.

It was shown by Thunberg [1920-21] that, if glutamic acid is added to washed frog muscle in presence of methylene blue, the methylene blue is reduced. Alanine alone of the other amino-acids used gave a similar but much slower decoloration. Thunberg postulated oxidative deamination. A little later, Abderhalden and Wertheimer [1921] found that addition of glutamic acid increases the oxygen uptake of red blood cells, muscle and kidney. The

other amino-acids tried had no effect; aspartic acid was tested on the red cells, though not on muscle. Ahlgren [1924] showed that, although washed frog's muscle does not activate aspartic acid, yet in the presence of unwashed muscle the reduction of methylene blue is accelerated by addition of aspartic acid. The appearance of an equilibrium point at partial reduction of methylene blue was reminiscent of the behaviour of malic or fumaric acid, and he was of opinion that hydrolytic deamination with formation of malic acid took place. Neither Thunberg nor Ahlgren investigated the question of ammonia formation. As we have seen, the results of the present work show that no simple deamination process occurs, either under aerobic or anaerobic conditions. This agrees with the experience of several workers who have found that sterile tissues or tissues with toluene have no power of producing ammonia from amino-acids. For example, Levene and Meyer [1913, 1, 2] were unable to find ammonia formation when aspartic acid, leucine, glycine or alanine was added to sterile leucocytes or kidney tissue. Luck [1924], using suspensions of various mixed tissues with toluene and hydrolysed gelatin or caseinogen as substrate, found no evidence for the formation of ammonia at the expense of free amino-nitrogen. That ammonia formation may take place in some cases on addition of amino-acids to sterile tissue has, on the other hand, been shown by Patey and Holmes [1929] for glycine in the presence of washed kidney. That in this case also there is not a straightforward oxidative deamination is shown by the fact that, although oxygen is necessary for the reaction, an oxygen absorption corresponding to the ammonia production could not be demonstrated.

As a hypothesis which would not contradict the facts described in this paper we might consider the possibility of the linkage of the NH_2 -group of the glutamic acid with some reactive carbohydrate residue in the muscle; when oxidation and splitting off of the nitrogen with production of succinic acid occurs, the nitrogen is left in combination with this carbon chain, a new α -amino-acid thus being formed.

It is well known that the oxidation of amino-acids *in vitro* may take different courses according to the presence or absence of carbohydrate substances. Fearon and Montgomery [1924] have pointed this out for the oxidation of glycine with dilute permanganate; more cyanic acid is formed and less ammonia when formaldehyde or glucose is present, and Fearon suggests that this result is due to combination between the NH_2 -group of the glycine and the aldehyde radical.

As regards the ammonia production by tissues, several workers have found carbohydrate or similar compounds to have an inhibitory effect upon this. Warburg, Posener and Negelein [1924], using rat carcinoma and retina, showed that both the anaerobic and the aerobic formation of ammonia were reduced to about one-tenth of their value by addition of glucose. Meyerhof, Lohmann and Meier [1925] found that the diaphragm muscle of starved rats produces ammonia in aerobic conditions, and this ammonia production is

greatly lessened by adding glucose, lactic acid or pyruvic acid. Watchorn and Holmes [1927] found less ammonia and urea formed during growth of tissue cultures when the medium contained glucose; and Patey and Holmes [1929], using washed kidney tissue, found that the aerobic (though not the anaerobic) ammonia production was much less in presence of glucose. The ammonia production from added glycine was not affected. If, in the case of glutamic and aspartic acids, in muscle, the presence of carbohydrate or its breakdown products prevents the appearance of free ammonia, then this prevention goes on anaerobically as well as in oxygen. This behaviour of certain carbon compounds in lessening ammonia formation is usually regarded as indicating "protein-sparing action"; in the present case, where it is postulated that one amino-acid is formed from another, part of the latter being oxidised, it would more correctly be described as "nitrogen-sparing."

It has been suggested by Thunberg [1920-21] that succinic acid might arise by removal of hydrogen atoms from two acetic acid molecules, and condensation of the residues. If this were the case, succinic acid might play an important part in fat metabolism, and also, as Wieland [1922] has pointed out, in carbohydrate metabolism. Lactic acid may give rise to pyruvic acid, and the latter by decarboxylation and oxidation to acetic acid. Very recently it has been shown by Butkewitsch and Fedoroff [1929] that, in presence of the mould *Mucor stolonifer*, acetic acid may be converted, to the extent of 15-30 %, into succinic and fumaric acids.

It has never, however, been possible to show that acetic acid can give rise to succinic acid in muscle. With unwashed muscle, Clutterbuck [1927] failed to find any increase in malic acid following upon incubation with acetate in oxygen; and I have similarly failed to find any increase with washed muscle. This does not prove that the condensation does not take place; in unwashed muscle a sufficiency of acetic acid may always be present, so that addition of more has no effect, while with washed muscle some part of the enzyme system responsible may have been removed.

Clutterbuck and Raper [1925, 1] have shown that γ -oxidation of fatty acids may take place with hydrogen peroxide *in vitro*, and they suggest that succinic acid may be formed in this way in the body; their experiments, however, on injecting β -phenylhexoic acid, gave negative results, no phenylsuccinic acid appearing in the urine [1925, 2].

In conclusion, it may be said that, although formation of succinic acid from glutamic and subsequent oxidation of the succinic acid is probably a part of muscle metabolism, yet it is not suggested that this is the only origin of the succinic acid in the body, or that the only function of the "succinoxydase" is to oxidise an intermediate stage in the breakdown of these amino-acids. Under anaerobic conditions, succinic acid formation seems to be a self-hindered reaction; under aerobic conditions, the acid is oxidised away and its formation cannot be measured; it may therefore play some as yet undetected part in carbohydrate and fat metabolism *in vivo*.

SUMMARY.

1. It has been shown that glutamic and aspartic acids, when added to minced muscle under anaerobic conditions, give rise to an increased amount of succinic, fumaric, and malic acids. The maximum amount of extra acid obtained was about 37 % of the equivalent of the amino-acids when both were added together; about 15 % when aspartic acid alone was added; and about 11 % when glutamic acid alone was added. From the mixed amino-acids or from aspartic acid alone, malic and fumaric as well as succinic acid were formed; from glutamic acid only succinic.

2. Ammonia was not produced during this formation of succinic, fumaric, and malic acids; nor could any increase of urea, uric acid, or amide-nitrogen be found. The amino-nitrogen (estimated by Van Slyke's method) at the end of the experiments was equal to that of the controls plus that of the added amino-acid.

3. An increased oxygen uptake was found when glutamic or aspartic acid was added to minced muscle. This uptake usually corresponded to one-half or two-thirds of the calculated amount for complete oxidation. The other amino-acids tried (glycine, alanine, valine, leucine and tyrosine) had an inhibitory effect on the muscle's own respiration.

4. Under aerobic conditions traces of malic and fumaric acids were found after addition of glutamic and aspartic acids. No change in the soluble nitrogen fractions was found.

5. A method for estimation of aspartic acid was worked out, and by its aid it was found that after incubation with muscle in air or oxygen one-half to two-thirds of the aspartic acid had disappeared.

6. It is suggested that possibly a combination of the amino-group with some reactive carbohydrate residue takes place; then when splitting and oxidation occur the amino-group is retained in the form of a new amino-acid.

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